

AWARD NUMBER: W81XWH-13-1-0245

TITLE: Assessing the Mechanisms of MDS and its Transformation to Leukemia in a Novel Humanized Mouse

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REPORT DATE: September 2014

TYPE OF REPORT: Annual Progress Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE September 2014		2. REPORT TYPE Annual Progress Report		3. DATES COVERED 15 Aug 2013 – 14 Aug 2014	
4. TITLE AND SUBTITLE Assessing the Mechanisms of MDS and its Transformation to Leukemia in a Novel Humanized Mouse				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-13-1-0245	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Stephanie Halene  E-Mail: Stephanie.halene@yale.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Yale University  New Haven, CT 06520-8021				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT The overall goal of this project is to develop a model for the study of myelodysplasia (MDS), an acquired bone marrow failure syndrome in the aging population and in cancer survivors. MDS is inherently difficult to study. MDS stem cells cannot be grown in culture and in vivo models are thus the gold standard. However, MDS stem cells are diseased and fail to efficiently engraft in current immunodeficient mouse models. We have optimized engraftment of normal adult HSPC into MISTRG. We have determined optimal cell preparation, cell number, pre-engraftment irradiation dose, transplantation route and recipient age. We have optimized the analysis of engrafted humanized mice. We have successfully transplanted primary MDS bone marrow cells into MISTRG mice with successful engraftment and replication of the donor disease status. MISTRG humanized mice tolerate chemotherapy and thus represent an ideal model to study disease progression.					
15. SUBJECT TERMS Myelodysplasia, Bone marrow failure, Humanized mice, Xenotransplantation					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT  Unclassified	18. NUMBER OF PAGES  11	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT Unclassified	b. ABSTRACT Unclassified	c. THIS PAGE Unclassified			19b. TELEPHONE NUMBER (include area code)

## Table of Contents

	<u>Page</u>
<b>1. Introduction.....</b>	<b>1</b>
<b>2. Keywords.....</b>	<b>1</b>
<b>3. Overall Project Summary.....</b>	<b>1</b>
<b>4. Key Research Accomplishments.....</b>	<b>7</b>
<b>5. Conclusion.....</b>	<b>7</b>
<b>6. Publications, Abstracts, and Presentations.....</b>	<b>7</b>
<b>7. Inventions, Patents and Licenses.....</b>	<b>n/a</b>
<b>8. Reportable Outcomes.....</b>	<b>n/a</b>
<b>9. Other Achievements.....</b>	<b>n/a</b>
<b>10. References.....</b>	<b>8</b>
<b>11. Appendices.....</b>	<b>n/a</b>

## Progress report

**Title:** Assessing the Mechanisms of MDS and its Transformation to Leukemia in a Novel Humanized Mouse

### Study Site and Personnel:

Yale University School of Medicine

Investigators:

PI: Stephanie Halene, M.D.

Mentor: Diane S. Krause, M.D., Ph.D. (no funding requested)

Collaborator: Richard A. Flavell (no funding requested)

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Research Assistant: Ashley Taylor

Postdoctoral Associate: Yang Liang

Animal Use: Xenotransplantation Studies; IACUC # 11396 approved

Human Subjects: Tissue collection; IRB # 12642 approved

### Introduction:

The overall goal of this project is to develop a model for the study of myelodysplasia (MDS), an acquired bone marrow failure syndrome in the aging population and in cancer survivors. MDS is inherently difficult to study. MDS stem cells cannot be grown in culture and *in vivo* models are thus the gold standard. However, MDS stem cells are diseased and fail to efficiently engraft in current immunodeficient mouse models. We here propose to develop an MDS *in vivo* model using a novel immunodeficient mouse, MISTRG, engineered to express human growth factors instead of murine growth factors, driven by the endogenous murine control elements in a temporally and spatially physiologic way. [1]

### Keywords

Myelodysplasia  
Bone marrow failure  
Humanized mice  
Cytokine knockin  
Xenotransplantation

### Overall project summary

The progress report summarizes our work during the first year of the funding period. The proposed studies seek to develop a xenotransplantation model for the study of myelodysplasia (MDS). We have optimized engraftment of normal adult HSPC into MISTRG. We have transplanted primary MDS bone marrow cells into MISTRG mice with successful engraftment and replication of the donor disease status. Further studies to determine treatment response, disease mechanism, clonal evolution, and progression to AML are under way as proposed for the second year of the funding period.

Task 1: IRB and HRPO review and approval for studies involving human subjects and IACUC and ACURO review and approval for animal use:

Local Internal Review Board and DoD regulatory review and approval of the human subject protocol to harvest bone marrow, peripheral blood, and buccal swab samples from patients has been obtained.

Local IACUC and DoD regulatory review and approval for the use of animals (mice) has been obtained.

Task 2. Optimize transplantation of primary MDS bone marrow cells into MISTRG mice: Optimize transplantation of primary MDS bone marrow cells into MISTRG mice by modulating pre-transplant irradiation doses, cell numbers and preparation, transplant route, and age of murine recipients

Task 2a. Transplantation of healthy adult stem cells.

The goal is to achieve multilineage engraftment levels of human cells  $> 10\%$  in the mouse bone marrow with high viability of recipients after transplantation.

We have based our studies with adult hematopoietic stem and progenitor cells (HSPC) on our prior studies with fetal liver derived HSPC. To assess successful engraftment, we have analyzed engraftment in peripheral blood (PB) and in bone marrow (BM) and spleen at 10-15 weeks after transplant.

We have analyzed engraftment by flow cytometric differentiation of human from murine cells and by analyzing human cell subsets based on lineage markers.

(i) Cell numbers and preparation:

Fetal liver CD34<sup>+</sup> cells are highly proliferative and as few as 3,000 CD34-selected cells can engraft humanized mice. Engraftment occurs in 100% of mice with engraftment levels of  $>90\%$  in mouse bone marrow when 50,000 fetal liver CD34-selected cells are engrafted. Viability of mice engrafted at  $>90\%$  is compromised due to development of anemia with viability of  $\sim 50\%$  8 weeks after transplant. Thus engraftment levels of  $\sim 50\%$  are desirable due to greater survival and sufficiently high engraftment levels to study diseases.

We transplanted mice with 100,000 – 150,000 adult CD34<sup>+</sup> cells and compared engraftment levels in MISTRG mice versus NSG mice, the current gold standard for xenotransplantation assays:

Engraftment levels were significantly higher in MISTRG mice than in NSG mice. In peripheral blood huCD45<sup>+</sup> cells comprised  $3.05 \pm 0.7\%$  of all CD45<sup>+</sup> cells in MISTRG and  $0.26 \pm 0.05\%$  of all CD45<sup>+</sup> cells in NSG ( $p < 0.05$ ). In bone marrow human CD45<sup>+</sup> cells comprised  $28.2 \pm 3.7\%$  in MISTRG and  $2.0 \pm 0.6\%$  in NSG ( $p < 0.0001$ ).

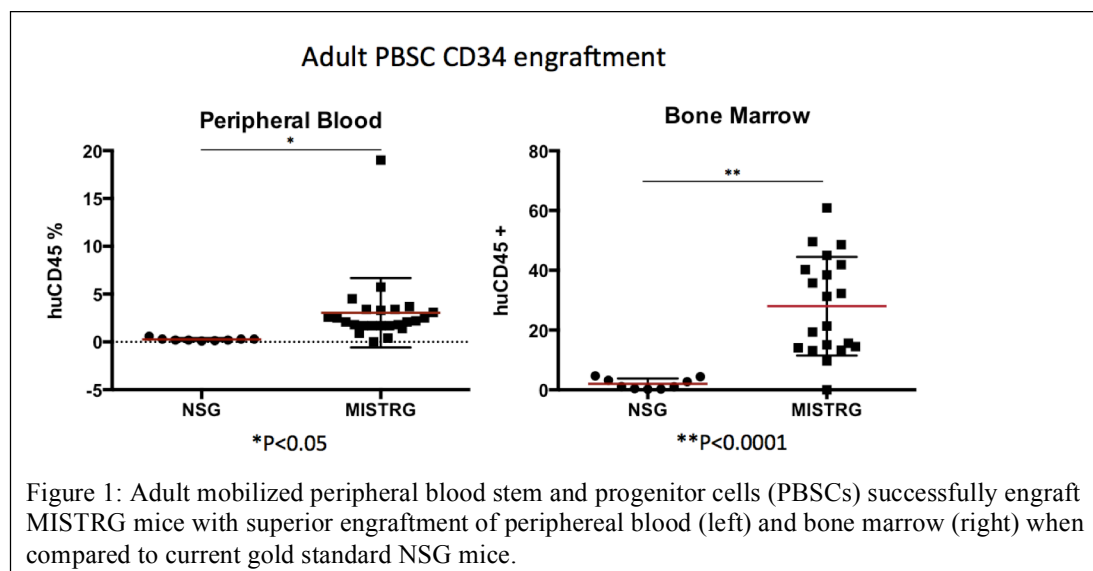


Figure 1: Adult mobilized peripheral blood stem and progenitor cells (PBSCs) successfully engraft MISTRG mice with superior engraftment of peripheral blood (left) and bone marrow (right) when compared to current gold standard NSG mice.

(ii) Pre-transplant Irradiation Dose:

MISTRG mice can engraft human cells without irradiation due to suboptimal murine stem cell maintenance secondary to humanization of cytokines.

Prior studies have established an irradiation dose of  $2 \times 150\text{cGy}$  for engraftment into newborn MISTRG mice. Mice do not exhibit toxicity from this irradiation dose and without transplantation viability is  $\sim 100\%$ . Since engraftment with 100,000 – 150,000 adult CD34<sup>+</sup> cells with prior irradiation of  $2 \times 150\text{cGy}$  gave optimal results with engraftment levels between 10-50% we chose  $2 \times 150\text{cGy}$  as the optimal for future studies.

(iii) Transplantation route:

Previous studies have established intrahepatic injection into newborn MISTRG mice as an optimal transplantation route. Intrahepatic injection has several advantages:

- 1) At time of birth hematopoiesis occurs in the bone marrow and newborn liver with progressive transition from the liver to the bone marrow. The bone marrow niche is primed to accept incoming stem cells, while the liver still continues to provide support for stem cells; this represents an optimal environment for human HSC engraftment into the humanized niche. The niche microenvironment is optimized for stem cell expansion to allow growth of the organism, providing optimal proliferation signals to engrafted stem cells.
- 2) Engraftment into newborn MISTRG pups is cost-effective. MISTRG litters comprise 7-12 pups which are weaned into cages of 3-5 mice/cage at 3-4 weeks of age. Viability post transplant is ~50-80%. Engraftment into adult mice retroorbitally requires mice ~6 weeks of age and intra-femorally ~8-12 weeks of age due to technical requirements. By injection into newborn mice, maintenance of mice pre-transplant can be avoided with savings of approximated \$100-200/litter of ~10 transplant recipients.
- 3) Intrahepatic transplantation into newborn mice is nearly atraumatic with up to 100% survival post procedure. Injection retro-orbitally into adult mice requires anesthesia, however, it is also rapid with near 100% survival. Intrafemoral injection requires prolonged anesthesia and is technically more difficult and time-consuming. Retro-orbital and intrafemoral injection are nevertheless viable options with positive engraftment when necessary.

(iv) Age of murine recipients: Based on our studies with fetal liver derived CD34+ cells have preferably used intrahepatic injection into newborn MISTRG mice.

This methodology has given reliable and multilineage engraftment of human adult CD34+ HSPCs.

Task 2b. Transplantation of MDS HSPCs.

We will use MDS hematopoietic stem and progenitor cells from primary patient bone marrow to establish optimal:

(i) Cell numbers and preparation:

Primary human bone marrow samples vary greatly in “quality” due to 1) patient’s disease and bone marrow status and 2) technical quality. Bone marrow for research purposes is taken at the time that the bone marrow procedure is performed for clinical purposes. Optimal sample delivery to the clinical labs is assured.

Cell numbers: primary MDS bone marrow samples vary between  $0.3 \times 10^6$  to  $1 \times 10^9$  cells/sample. Clinical factors determining cell number obtained are:

Bone marrow cellularity

Presence of absence of fibrosis

MDS subtype – lower grade MDS generally yields lower cell numbers than higher grade MDS.

Based on our studies, when  $>1 \times 10^7$  mononuclear cells are obtained xenotransplantation of ~5 recipients is feasible. Samples with  $<1 \times 10^7$  cells are unlikely to yield significant engraftment in a significant number of mice ( $\geq 5$ ).

Cell preparation: All bone marrow samples are ficoll to obtain the mononuclear cell fraction and cryopreserved in aliquots of  $1-5 \times 10^7$  cells/vial; at time of xenotransplantation samples are rapidly thawed and processed for transplantation.

Based on our studies and studies by Wunderlich et al.[2], we pre-incubate primary bone marrow samples with an anti-CD3 antibody to prevent engraftment of alloreactive T-cells. In MISTRG engraftment of T-cells is thus prevented.

- 1) Transplantation of unsorted bone marrow is feasible with pre-incubation of anti-CD3 antibody
- 2) T-cell depletion and engraftment of the CD3 negative fraction is feasible with or without pre-incubation of anti-CD3 antibody
- 3) CD34 selection and engraftment of the CD34-selected fraction gives optimal engraftment results with multilineage hematopoiesis (absent T-cells with pre-incubation with anti-CD3 antibody).

(ii) Pre-transplant Irradiation Dose:

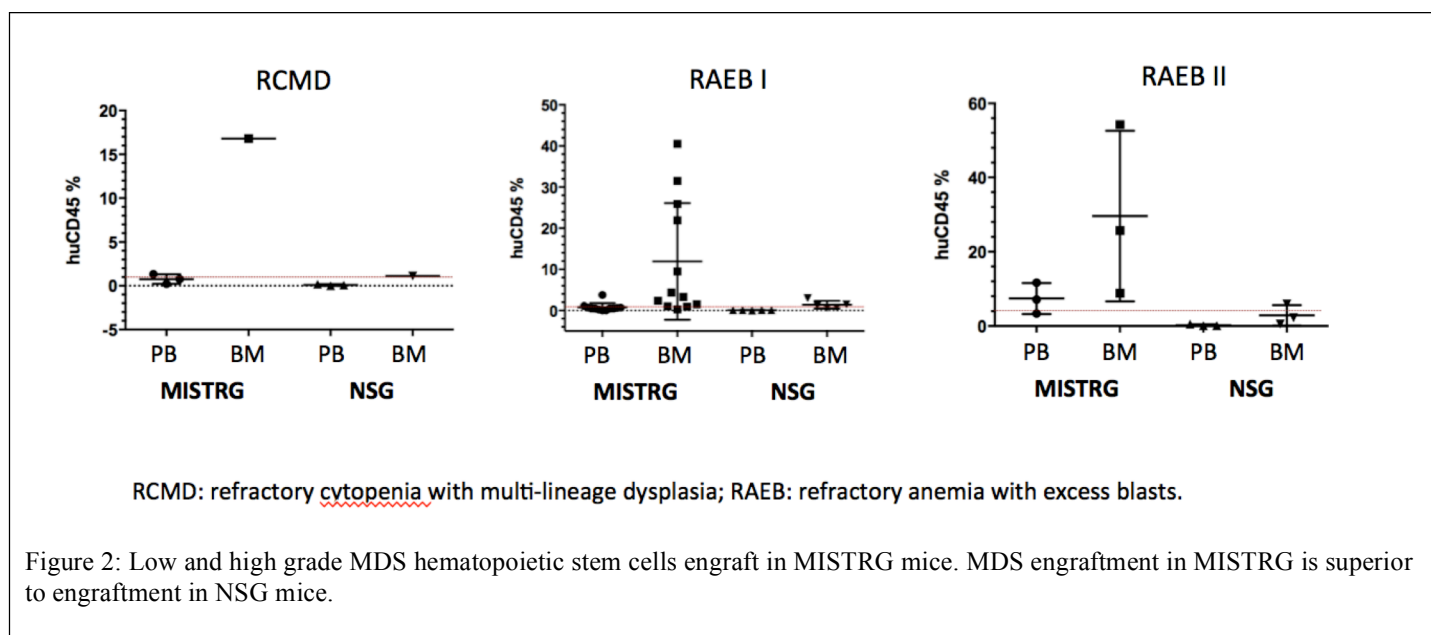
Based on studies with normal CD34+ HSPC engraftment a pre-transplant irradiation dose of 2x150cGy administered 4-8 hours apart was chosen.

(iii) Transplantation route:

Based on studies with normal CD34+ HSPCs intrahepatic engraftment into newborn MISTRG mice was chosen.

(iv) Age of murine recipients: Based on our studies with fetal liver derived CD34+ cells and adult HSPCs intrahepatic engraftment into newborn mice was chosen. The major risk of intrahepatic injection in newborn mice is rejection by the mom. We have extended the injection time from postnatal day 1 to day 3. MISTRG mice successfully engraft up to day 3 postnatally.

Based on these techniques we have established an efficient and reliable MDS xenotransplantation model. Examples of successful MDS xenotransplantation results are given in Figure 2:

Task 3. Study MDS disease progression MISTRG mice (mths 10-24):

Study disease progression in MISTRG mice, such as clonal evolution and progression to AML

These studies are underway.

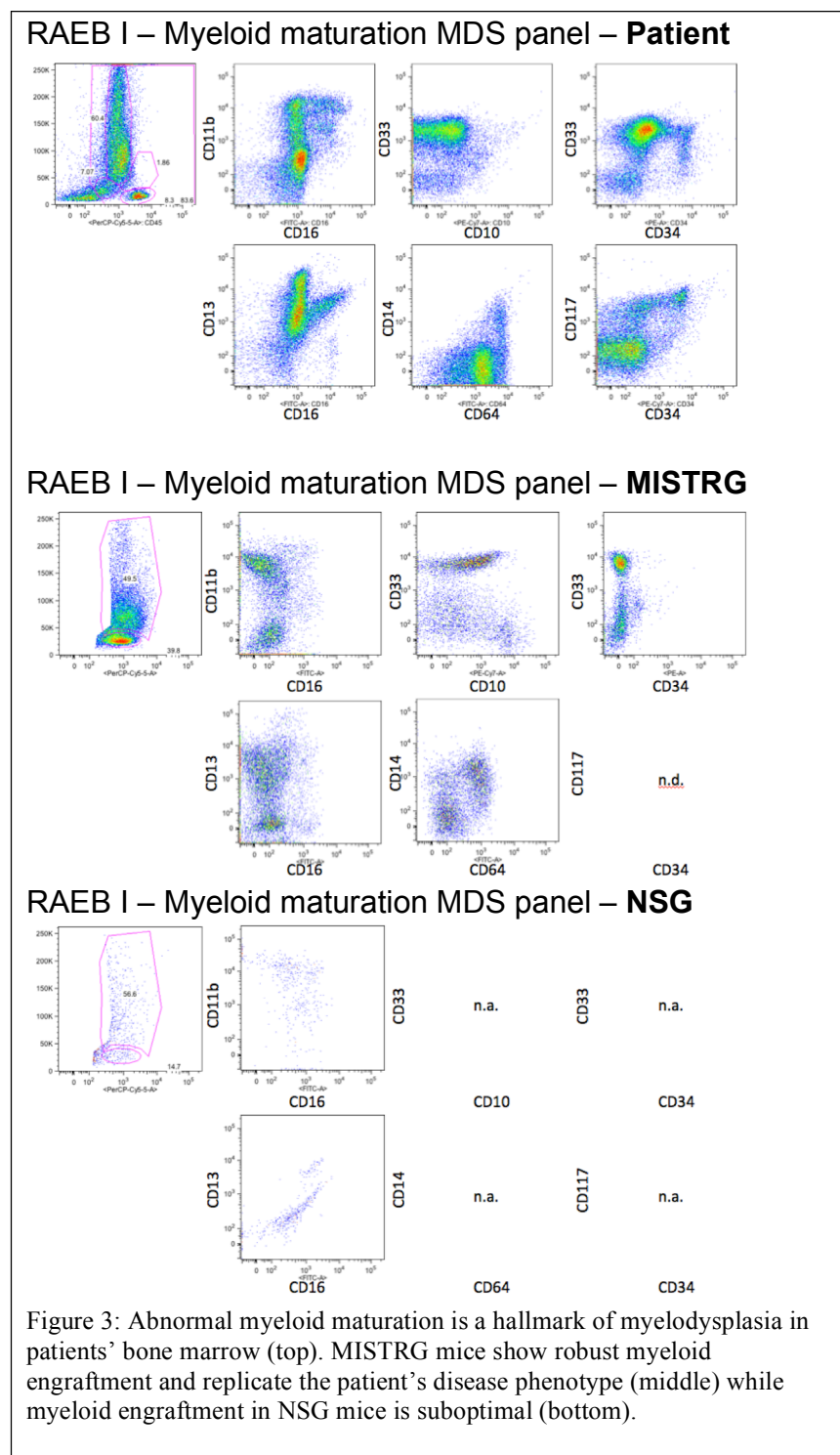
Task 3a. Analyze MDS-engrafted primary and secondary MISTRG recipients for disease phenotype and clonality.

We will transplant MDS derived HSPC into MISTRG mice with the protocol established from task 2a. and 2b. We will transplant MDS derived HSPC from patients with different subtypes of MDS (according to the World Health Organization (WHO) classification). Due to the heterogeneity of the disease we will transplant as many patient samples as possible to establish a solid model and to understand the different subtypes of the disease, contribution of different genetic and epigenetic abnormalities, and patient history.

MISTRG mice replicate the disease phenotype in patients.

We engrafted MISTRG and NSG mice with primary bone marrow cells from MDS patients. We engrafted CD34-selected cells, T-cell depleted cells, total bone marrow with pre-incubation with anti-T-cell antibody to prevent acute graft versus host disease.

MISTRG replicate the disease phenotype in regards to myeloid differentiation and blast percentage encountered in human samples.

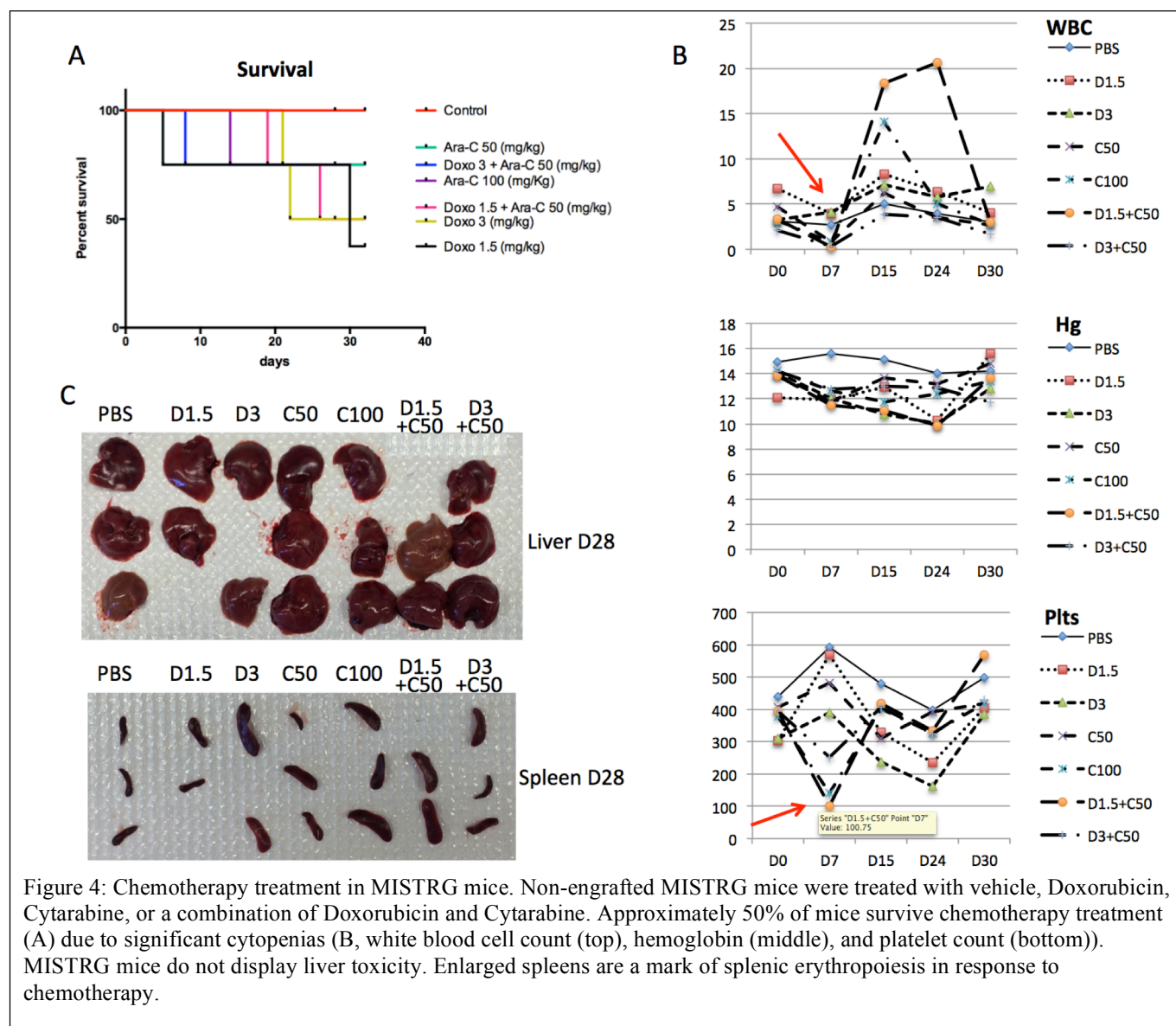


**Task 3b. Establish treatment protocols in (i) non-engrafted and then (ii) engrafted MISTRG mice .**

**Drug treatment:** We have successfully established a treatment protocol using standard chemotherapy in MISTRG mice.

As opposed to NSG mice, MISTRG mice do not carry an inherent DNA repair defect, thus allowing the use of cytotoxic drugs. First studies have been performed in non-engrafted mice to assess toxicity of chemotherapeutic

agents in MISTRG mice. MISTRG mice tolerated cytarabine (50mg/kg vs 100mg/kg, daily x 5 days) and doxorubicin (1.5mg/kg vs 3mg/kg, daily x 3days) alone and in combination without significant liver toxicity unlike NSG mice. However, main toxicity in MISTRG mice is hematopoietic toxicity and survival after chemotherapy administration is approximately 50%. Additional studies are under way.



#### Task 4. Develop a model to study MDS “disease-driving” mutations (mths 1-24):

Study individual mutations identified in our primary MDS samples by introduction of these mutations into human CD34+ stem and progenitor cells followed by in vitro and in vivo analysis

##### 2a. Generation of inducible lentiviral vectors and *in vitro* analysis of transduced CD34+ cells.

These studies are under way.

Research Timeline	Year 1				Year 2			
Review and approval of studies involving human subjects (IRB; HRPO) and animals (IACUC; ACURO)	✓							
<b>Aim 1.</b> Optimize transplantation of primary MDS bone marrow cells into MISTRG mice		✓	✓	✓				
Aim 1.1 Transplantation of healthy <u>adult</u> stem cells		✓	✓					
Aim 1.2 Transplantation of MDS HSC			✓	✓				
<b>Aim 2.</b> Study MDS disease progression in MISTRG mice								
Aim 2.1a Analyze MDS-engrafted primary and secondary MISTRG recipients for disease phenotype and clonality				✓				
Aim 2.1b Establish treatment protocols in non-engrafted and then engrafted MISTRG mice		✓	✓					
Aim 2.2 Develop a model to study MDS “disease-driving” mutations	✓	✓	✓	✓				

**Key Research Accomplishments:**

- Optimal transplantation technique into MISTRG for adult HSPCs has been established in regards to cell preparation, cell number, pre-transplant irradiation, and intrahepatic injection in d1- d3 old pups.
- Engraftment of adult HSPCs in MISTRG mice is superior to standard immunosufficient mice.
- Patient MDS bone marrow derived HSPCs successfully engraft MISTRG mice. An optimal transplantation model has been established.
- MISTRG give rise to multilineage engraftment and successful myeloid maturation.
- Engraftment of MDS HSPCs in MISTRG replicates the patient’s immunophenotype.
- MISTRG mice are tolerant of standard chemotherapy treatments, a prerequisite to determine clonal evolution in response to treatment.

**Conclusions**

MISTRG mice represent a novel model for xenotransplantation of human hematologic diseases, in particular MDS. MDS xenotransplantation in MISTRG represents the first *in vivo* model for the study of MDS other than the patient himself and will aid in therapy development.

**Publications, Abstracts, Presentations:**

## a. Abstracts:

- 12th International Symposium on Myelodysplastic Syndromes (MDS), Berlin, Germany, May 2013 "Modeling Myelodysplasia in a Novel Humanized Mouse"

## b. Presentations:

- Yale Cardiovascular Research Meeting, April, 2014, “Xenotransplantation Models for MDS”
- Yale Comprehensive Cancer Center, January 2014, “Splicing factor mutations in MDS and MDS xenotransplantation”

**Inventions, Patents, and Licenses**

n/a

**Reportable Outcomes**

n/a

**Other achievements**

n/a

**References:**

1. Rongvaux, A., et al., *Development and function of human innate immune cells in a humanized mouse model*. Nat Biotechnol, 2014. **32**(4): p. 364-72.
2. Wunderlich, M., et al., *OKT3 prevents xenogeneic GVHD and allows reliable xenograft initiation from unfractionated human hematopoietic tissues*. Blood, 2014. **123**(24): p. e134-44.